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DEVELOPMENT OF AN AUTONOMOUS PATHOGEN DETECTION SYSTEM

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ABSTRACT

An Autonomous Pathogen Detection System (APDS) is being designed and evaluated for use in domestic counter-terrorism. The goal is a fully automated system that utilizes both flow cytometry and polymerase chain reaction (PCR) to continuously monitor the air for BW pathogens in major buildings or high profile events. A version 1 APDS system consisting of an aerosol collector, a sample preparation subsystem, and a flow cytometer for detecting the antibody-labeled target organisms has been completed and evaluated. Improved modules are under development for a version 2 APDS including a Lawrence Livermore National Laboratory-designed aerosol preconcentrator, a multiplex flow cytometer, and a flow-through PCR detector.

INTRODUCTION

Lawrence Livermore National Laboratory (LLNL) has an ongoing program to design, fabricate and field demonstrate a fully Autonomous Pathogen Detection System (APDS). This will be accomplished by integrating a flow cytometer and real-time polymerase chain reaction (PCR) detector with sample collection, sample preparation and fluidics to provide a compact, autonomously operating instrument capable of simultaneously detecting multiple pathogens and/or toxins. The APDS will be designed to operate in fixed locations, where it continuously monitors air samples and automatically reports the presence of specific biological agents. The APDS will utilize both multiplex immuno and nucleic acid assays to provide 'quasi-orthogonal', multiple agent detection approaches to minimize false positives and increase the reliability of identification. The APDS is targeted for domestic applications in which (1) the public is at high risk of exposure to covert releases of bioagent such as in major subway systems and other transportation terminals, large office complexes, and convention centers; and (2) as part of a monitoring network of sensors integrated with command and control systems for wide-area monitoring of urban areas and major gatherings (e.g., inaugurations, Olympics, etc.). In this latter application there is potential that a fully developed APDS could add value to DoD monitoring architectures.

Technical advancements across several fronts must first be made in order to realize the full extent of the APDS. A phased approach, with increasing detection and identification capability, has been employed in the design, construction, and testing of each evolutionary version of the APDS. The version 1 system, APDS-I, which provides a single-plex immunoassay using flow cytometry, has been completed and field tested. Work is in progress on advanced modules for the second version of the APDS, APDS-II, to provide the capabilities for *automated multiplex* immunoassays and *flow-through PCR*.

DEVELOPMENT AND EVALUATION OF APDS-I

This system incorporates an aerosol collector from Research International (RI), a custom-built fluidics system, and a MicroCyte flow cytometer. The fluidic system is capable of mixing and dispensing three different reagents for incubation with the sample before delivery to the flow cytometer. The control system utilizes LabView for instrument operation, for acquisiton and data storage, and to automatically call positive samples. A direct-labeling immunoassay for B.g. was developed for the MicroCyte flow cytometer, and this assay was demonstrated for dilutions of B.g. spores in buffer, and spores in aerosol collector fluid. Figure 1 shows the completed APDS-I.

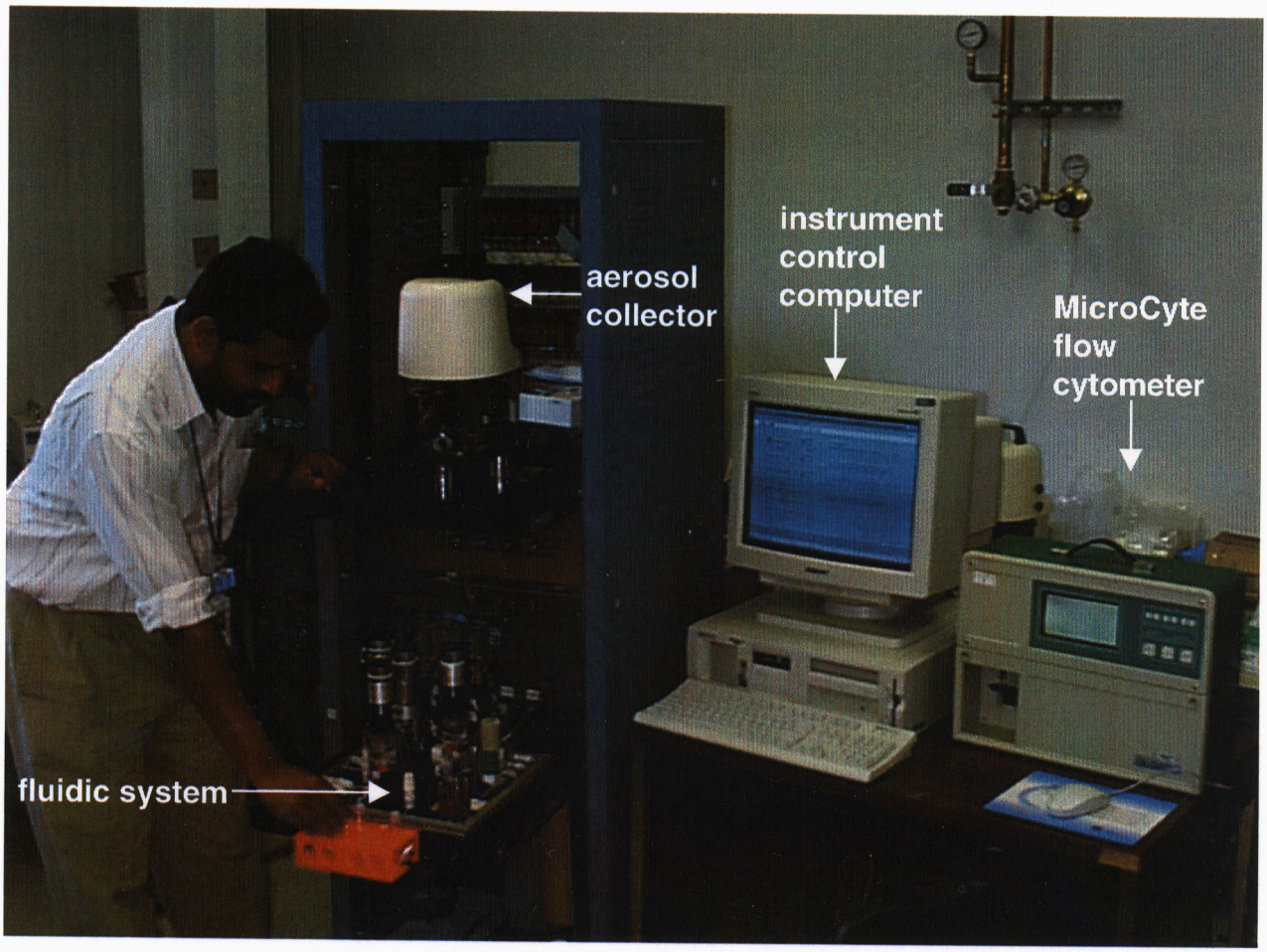

Figure 1: Version 1 Autonomous Pathogen Detection System. Minimal effort was expended to develop an integrated instrument for this proof-of-concept version of the APDS.

The integrated APDS-I instrument was transported to the Pacific Northwest National Laboratory Wind Tunnel Facility to benchmark the overall performance of the system. The APDS-I was challenged with a series of B.g. releases over a wide range of concentrations for a three day period. Highlights of these tests include a 12-hour fully automated run with results reported every 20 min.; successful detection of B.g. down to concentration levels of 50 spores per liter; and system response proportional to the aerosol spore concentration. Figure 2 shows an example of the data obtained from these wind tunnel tests.

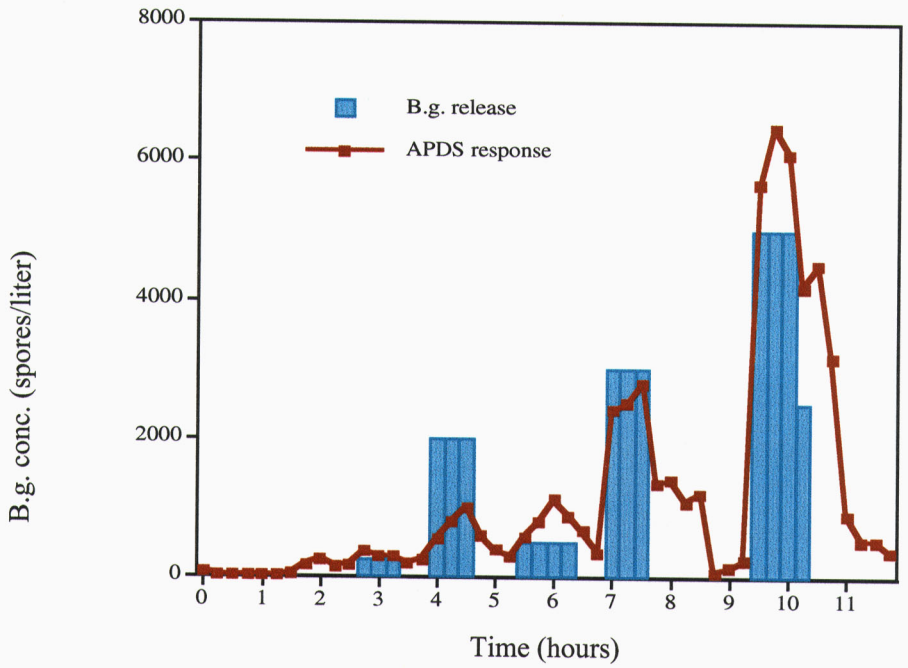

Figure 2: A 12-hour continuous APDS run at the PNNL wind tunnel

EVALUATION OF NEW MODULES FOR APDS-II

We have adopted a modular approach in our development of the APDS-II. Separation of the modules is based on their functionality (i.e., aerosol collector, sample preparation fluidics, nucleic acid assay, and multiplex flow cytometry immunoassay). This approach allows us the flexibility to develop and optimize each module before it is integrated into the final autonomous system. The first objective is to develop and demonstrate an integrated instrument containing an aerosol collector, sample preparation module (fluidics), and multiplex immunoassay. In this approach, the aerosol collected sample is added to a collection of microbeads. Each color of microbead contains a capture assay that is specific for a given bioagent. Fluorescent labels are then added to identify the presence of each agent on the bound bead. Each optically encoded and fluorescently labeled microbead is then individually read in the flow cytometer. Subsequently, in-line nucleic acid recognition (flow-through PCR) will be added to the system.

Aerosol collector

The aerosol collector used in APDS-I was the SASS-2000 cyclone sampler (Smart Air Sampler System, Research International). A custom hybrid sampler has been developed that utilizes an LLNL designed virtual impactor for selectively sampling a given particle size range coupled to a SASS-2000 aerosol collector. The performance of this LLNL/SASS Hybrid was compared with two other collectors, the SASS-2000 alone, and the SCAEP (Space Charged Atomizing Electrostatic Precipitation, Team Technologies) model PM-1B. The industrial standard All Glass Impinger; AGI-30 (Andersen Laboratories) was used as the reference sampler. Side-by-side system performance comparisons conducted in two different field trials at the Harry Reid Center for Environmental Studies, University of Nevada-Las Vegas (UNLV).

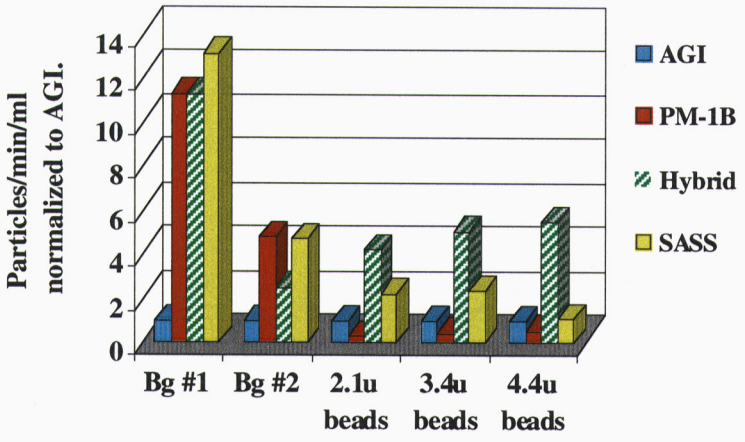

Figure 3: Side by side comparison of aerosol collectors from UNLV field trial

The results indicated that the LLNL/SASS Hybrid has the best overall collection rate for the different samples and particle sizes tested (Figure 3). Custom modifications to the LLNL/SASS Hybrid instrument for optimum adaptation to the APDS-II are nearing completion. Some of the more important upgrades to the LLNL/SASS Hybrid, include, increased air flow, variable particle size selection, smaller packaging, onboard particle counter, ruggedized design, and autonomous Labview-based control system.

Sample preparation (fluidics)

The multiplex immunoassay protocol will be used to determine the sequence of actions performed by the sample preparation module. Since engineering of the sample preparation module and assay development are necessarily parallel efforts, we defined a generalized set of operations for the sample preparation module that is flexible enough to cover virtually any defined protocol. These operations include:

- extracting a portion of the collected sample volume from the aerosol collector
- capturing the sample with antibody-labeled microbeads
- labeling the samples with fluorescent reporter antibodies
- separating, washing, and pre-concentrating microbeads to improve assay performance and prevent cross-contamination between measurements
- flowing the resulting mixture to the flow cytometer for analysis

The approach used in APDS-I was neither flexible nor modular enough to accommodate the added complexity of operations required for APDS-II. Based on experience with our previously developed in-line PCR system and some early bench testing, we adopted a technique advocated by a collaborator (Global FIA, Gig Harbor, WA) using a sequential injection analysis (SIA) platform. The basic components of this platform include: a carrier fluid; a syringe pump; a holding and mixing coil; a multi-port selection valve; reagent/sample storage; and separation cell (Figure 4). The carrier fluid is used to draw and pump fluids sequentially through the various sample ports on the selection valve. Aliquots of air are used to spatially separate the carrier from reagent and sample volumes, greatly minimizing the chance of cross-contamination. The holding/mixing coil serves to mix various assay components (i.e., sample, microbeads, reporter, etc.), to perform incubation (both heated and unheated coils are being tested), and to prevent contamination of the syringe pump. The selection valve serves as the interface between all components of the sample preparation unit, offering a flexible medium for changing and upgrading the various fluidic components. The separation cell is used to separate, wash, and/or preconcentrate the microbeads at various points in the multiplex immunoassay. A fluidics testbed with a simple serial based Labview control system and modified Global SIA hardware has been constructed to develop assay protocols.

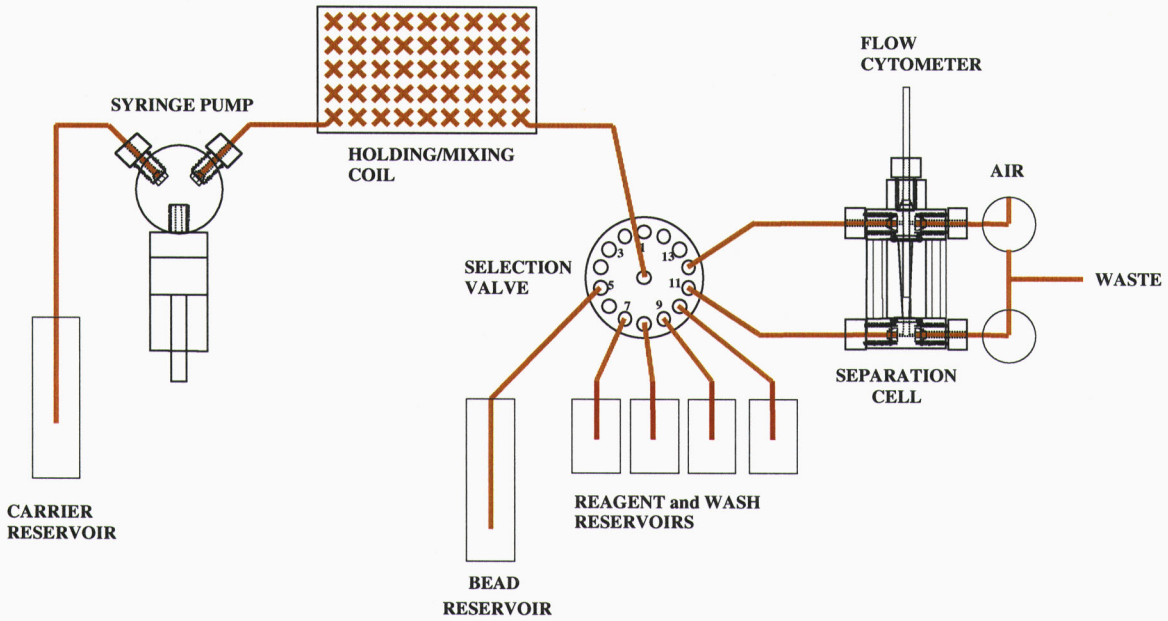

Figure 4: SIA based sample preparation module for APDS-II

Multiplex flow cytometer and immunoassays

A major advancement related to large-scale multiplex immuno-analysis is the recent release of the Luminex, model LX-100 flow cytometer. This instrument has a 100-plex target capability and constitutes the centerpiece of our development effort for incorporating multiplex flow cytometry into the APDS-II system. We have recently obtained samples of all 100 bead sets, and a mixture of all bead sets has been successfully analyzed at LLNL (Figure 5).

The current focus of our assay development is the demonstration of an automated 7-plex detection (four bioagent simulants, three controls) capability. The result shown in Figure 6 is close to achieving that goal. Bead sets were coated with specific antibodies for *Bacillus globigii* (Bg), *Erwinia herbicola* (Eh), bacteriophage (MS2), Ovalbumin (Ov), and selected fluorescent reporter dyes. We also coated two separate bead sets with reagents that provide internal positive and negative controls for each step in the sample preparation process. These controls will provide a continuous monitor of the APDS-II operation even if no target agents are detected.

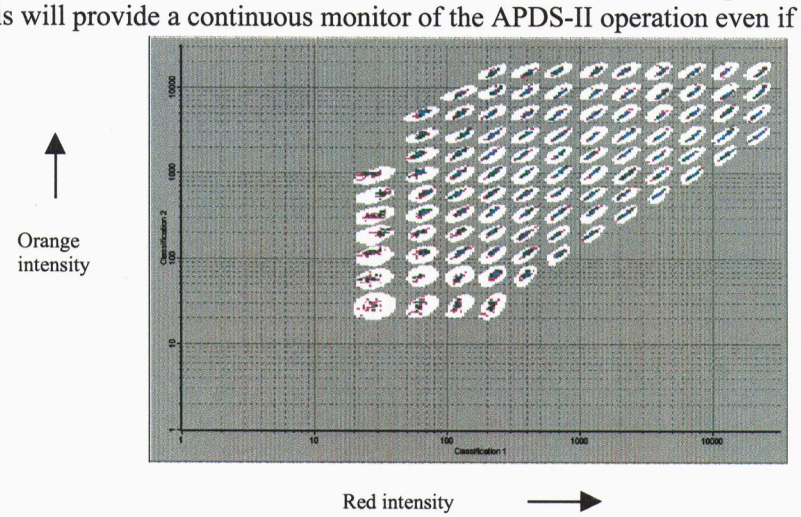

Figure 5: Dot plot of a mixture of 100 bead sets depicting adequate separation of each bead set

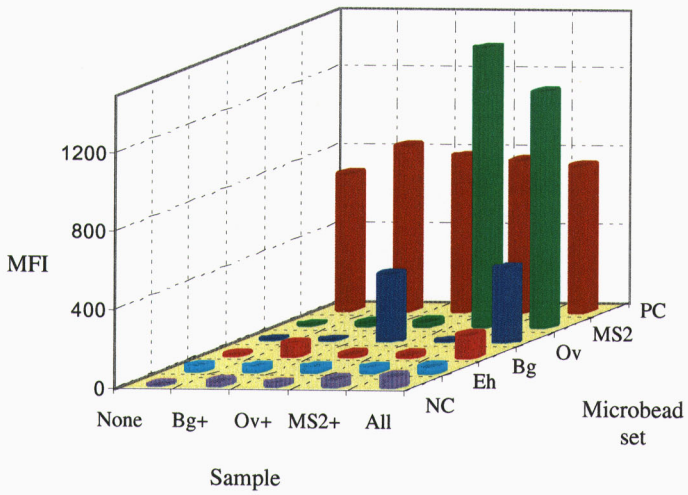

Figure 6: Luminex LX-100 analysis of 4 simulants using a 6-plex assay. Median fluorescent intensities (z-axis) of each microsphere set (y-axis) analyzed from 5 different samples (x-axis)

Flow-Through PCR

Flow-through PCR provides a method for conducting continuous, real-time nucleic acid assays. This technology will add an orthogonal detection technique to the multiplex immunoassay capability, thereby increasing the reliability of a positive identification and providing additional information regarding the origin and characteristics of a detected pathogen. The flow-through PCR system consists of a LLNL-designed, silicon-machined thermocycler mounted in-line with a sequential injection analysis system (Figure 7). The SIA system performs all necessary sample preparation functions (mixing of sample with PCR reagent components, etc.) and delivers the prepared PCR reagent/sample aliquot to the thermocycler unit. The thermocycler is designed with appropriate light sources and detectors to perform real-time TaqMan assays. After completion of the assay, the SIA system decontaminates the thermocycler chamber and all exposed fluid delivery tubes.

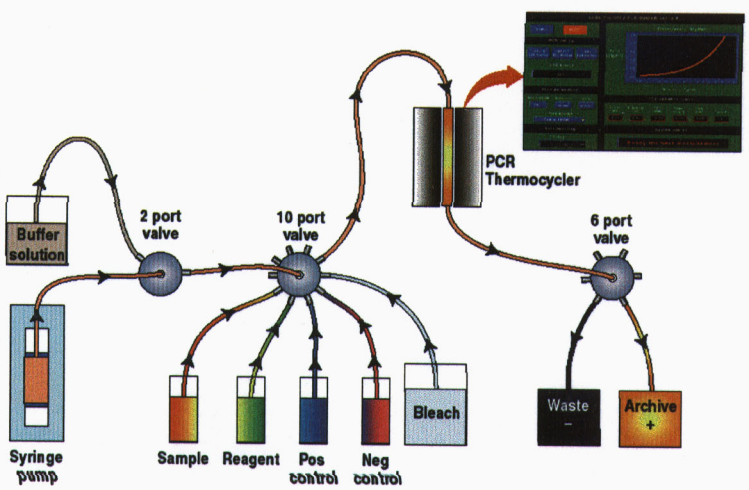

Figure 7: Flow-through PCR system

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The flow-through PCR system has undergone extensive characterization during FY00. We successfully demonstrated amplification in 10^6 cfu/ml of several different bacterial species, including Bacillus globigii (*B.g.*), Bacillus thuringensis (*B.t.*), and Erwinia herbicola (*E.h.*). Amplification with as little as 10 µl of sample (7µl reagent, 3µl sample) has been successfully performed. We have also determined that the chamber and sample port can be quickly decontaminated by flushing with small volumes of bleach, making possible repeated cycling of positive and negative controls through the same sample port.

System integration

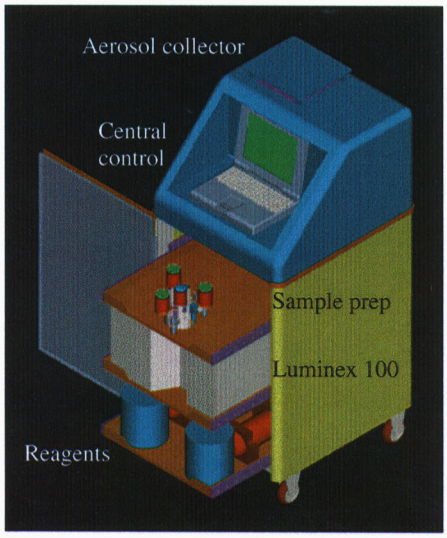

Figure 8: Integrated APDS system

We have completed a preliminary design for packaging and physically connecting the different components of the APDS-II system in a simple, "ATM" style chassis (Figure 8). Concerns such as heat transfer, vibration isolation, fluidic connections, flexibility, and user access are addressed in this design. Space for future modules, such as the in-line nucleic acid assay, has also been allocated in this chassis.

One of the most challenging tasks of the entire APDS development project is the unification of the separate components under a central control system. We chose National Instrument's Labview software as the common programming language for APDS, and have currently written software to control both the aerosol collector and sample preparation module. The largest component, reprogramming the LX100 multiplex flow cytometer in Labview, is currently in progress. The graphical user interface (GUI) is complete and currently undergoing a second round of improvements.

CONCLUSION

The successful field test of APDS-I demonstrated the feasibility of fully automated continuous environmental monitoring for pathogenic organisms. The development of bead-based immunoassays for APDS-II provides the potential for large-scale multiplex analysis that can be easily adapted to new potential threat agents. Finally, incorporation of PCR-based nucleic acid analysis should greatly increase the reliability and specificity of agent identification.

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